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Calof AL, et al
Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium.
Ciba Found Symp. 1991;160:249-65; discussion 265-76.
PMID: 1752166; UI: 92090184.

Pixley SK, et al.
Cultured rat olfactory neurons are excitable and respond to odors.
Brain Res Dev Brain Res. 1990 Apr 1;53(1):125-30.
PMID: 2350878; UI: 90275756.

Pixley SK.
Characterization of olfactory receptor neurons and other cell types in dissociated rat olfactory cell cultures.
Int J Dev Neurosci. 1996 Nov;14(7-8):823-39.
PMID: 9010728; UI: 97163981.

Noble M, et al.
The growth of olfactory neurons in short-term cultures of rat olfactory epithelium.
Neurosci Lett. 1984 Mar 23;45(2):193-8.
PMID: 6728313; UI: 84220439.

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Maue RA, et al.
Preparation of isolated mouse olfactory receptor neurons.
Pflugers Arch. 1987 Jul;409(3):244-50.
PMID: 3627945; UI: 87316795.

Schubert D, et al.
Ontogeny of electrically excitable cells in cultured olfactory epithelium.
Proc Natl Acad Sci U S A. 1985 Nov;82(22):7782-6.
PMID: 2866512; UI: 86067989.

Wolozin B, et al.
Continuous culture of neuronal cells from adult human olfactory epithelium.
J Mol Neurosci. 1992;3(3):137-46.
PMID: 1320921; UI: 92329300.

Sosnowski JS, et al.
Chemical traumatization of adult mouse olfactory epithelium in situ stimulates growth and differentiation of olfactory neurons in vitro.
Brain Res. 1995 Dec 8;702(1-2):37-48.
PMID: 8846094; UI: 96255746.

Eustache I, et al.
Effects of insulin-like growth factors on organotypic cocultures of embryonic rat brainstem slices and skeletal muscle fibers.
Brain Res Dev Brain Res. 1994 Sep 16;81(2):284-92.
PMID: 7813048; UI: 95112382.

J Mol Neurosci (1992) 3:137–146

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# Continuous Culture of Neuronal Cells from Adult Human Olfactory Epithelium

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**Abstract.** Cells from the olfactory epithelium of adult human cadavers have been propagated in primary culture and subsequently cloned. These cells exhibit neuronal properties including: neuron-specific enolase, olfactory marker protein, neurofilaments, and growth-associated protein 43. Simultaneously, the cells exhibit nonneuronal properties such as glial fibrillary acidic protein and keratin, the latter suggesting properties of neuroblasts or stem cells. These clonal cultures contain 5–10% of cells sufficiently differentiated to show odorant-dependent cyclic adenosine 3′,5′-monophosphate (cAMP) or calcium-release responses when challenged with submicromolar concentrations of odorants. The potential of culturing neuronal cells from patients with neuropsychiatric disorders, such as Alzheimer's disease or schizophrenia, could enable the study of the pathophysiology of these neurons in the culture dish and allow new approaches to the study of mental illness.

The olfactory neurons are unique in their ability to regenerate into adult life. The neurons are continuously generated from stem cells located at the base of the epithelial layer. After lesion of the olfactory tract and subsequent death of the primary olfactory neurons, the regeneration occurs at a rate of 2–3% per day (Graziadei, 1973). The capability of these cells to divide has focused much effort on methods of maintaining these cells in culture (Coon et al., 1989; Goldstein et al., 1986; Pixley and Pun, 1990; Schubert et al., 1985). Cells from newborn rats with properties of neurons and that exhibit odorant responses have been propagated in culture (Coon, 1989). Similar cultures from rat fetuses produced small numbers of differentiated neurons (Schubert et al., 1985). The ability of these cells to regenerate indicates that they maintain the capacity to proliferate into adult life and suggests that olfactory neurons obtained from adults could also be propagated in cell culture.

Mammalian olfactory receptor cells are located in the epithelium of the upper nasal cavity just under the cribriform plate. As the neurons mature, they first send an axon to the olfactory bulb and the cell body migrates toward the surface of the epithelium extending a bulbous dendritic process with a ciliated structure, the olfactory vesicle, to the mucus surface (Graziadei, 1973). The axons of the olfactory neurons communicate directly with the central nervous system; hence, although not part of the central nervous system, these neurons are intimately associated with the central nervous system. These cells express a variety of neuronal antigens including neuron-specific enolase (NSE) and neural cell adhesion molecule (NCAM) (Calof and Chikaraishi, 1989). These cells also express a variety of molecules specific to the olfactory system, including olfactory marker protein (OMP), olfactory specific adenylate cyclase, and putative odorant receptors (Keller and Margolis, 1975; Bakalyar and Reed, 1990; Buck and Axel, 1991). The presence of cilia appears to be important, as shown by the localization of a putative odorant-related adenylate cyclase in the cilia of the olfactory epithelium (Bakalyar and Reed, 1990). These cells also express the epithelial marker keratin and a cytoskeletal protein of characteristic of immature cells, vimentin (Calof and Chikaraishi, 1989; Schwob et al., 1986).

We now show that cells exhibiting some of the characteristics of olfactory neurons can be propagated from explants of adult human olfactory epithelium harvested at autopsy. Because these cells are intimately connected with the central nervous

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system, these olfactory neuroblasts (ON) may also exhibit central nervous system disease-related pathophysiology, as has already been shown for Alzheimer's disease (Talamo et al., 1989). The ability to culture neuronal cells from adult humans with diagnosed neuropsychiatric illness opens a new avenue of research for the study these illnesses in cell culture.

## Materials and Methods

### Tissue acquisition

Olfactory epithelium was obtained from 11 human subjects at autopsy. The postmortem interval varied from 7 to 14 hours. The age of the donors range from 19 to 62 years. Following acquisition of the specimens, the fresh tissue was placed in L-15 medium for transport (Kischer et al., 1989).

### Cell Culture

Following a 1-hour incubation at room temperature in medium containing antibiotics and antimycotics, the tissue was dissected into pieces about 1 $mm^2$ and placed onto tissue culture dishes. Each piece was then covered with a drop of reconstituted basement membrane (RBM) (available as Matrigel, Collaborative Research) and incubated for at least 20 minutes at 37°C in order to allow the RBM solution to form a gel. The immobilized tissue was covered with 4506 medium, which has been shown to permit the propagation of olfactory neuroblasts from neonatal rat: Coon's modified Ham's F-12 medium, neuroblast formulation (Whittaker), supplemented with 6% fetal calf serum (Gibco), 150 μg/ml bovine hypothalamus extract, 50 μg/ml bovine pituitary extract, 1 μg/ml Na-insulin (Elanco), 5 μg/ml human transferrin, 10 nM hydrocortisone (Sigma), 40 pg/ml thyroxine, 2.5 ng/ml selenious acid, and 60 μg/ml gentamycin (Whittaker) (Coon et al., 1989). After 1–4 weeks of culture, cells began to grow out from the tissue. Colonies of morphologically identical cells were isolated using cloning cylinders and subsequently expanded. Monoclonal selection was performed in two ways. Cells that had been previously selected using cloning cylinders were plated at low dilution, allowed to multiply, and individual colonies were then isolated again using cloning cylinders. Alternatively, cells that had been previously selected using cloning cylinders were successively diluted to yield one cell or less per well. These single cell colonies were them allowed to multiply. Growth rates were monitored using a bromodeoxyuridine kit (Amersham) following the manufacturer's directions.

### Immunocytochemistry

Cells were fixed for at least 2 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100, and blocked with 5% nonfat dry milk. Dilutions of primary antibodies were 1 to 2000 for NSE (Polysciences), 1 to 10 for anti-neurofilament light chain (Boehringer Mannheim), 1 to 500 for anti-OMP (gift of F. Margolis) and 1 to 400 for anti-calcitonin gene-related peptide (Amersham) in PBS with 5% bovine serum albumin. Immunoreactivity was visualized with the Elite Peroxidase ABC kit (Vector) using a 2-hour incubation with the biotinlylated secondary antibody and a 1-hour incubation of the ABC–peroxidase complex, both at room temperature. Dilutions and diluents were according to manufacturers directions. 3,3'-Diaminobenzidine was used as the substrate for color development. For double-staining experiments, rhodamine- and flourescein-labeled secondary antibodies were used rather than an ABC peroxidase kit.

**Immunoblotting.** Polyacrylamide gel electrophoresis was performed using 10% acrylamide with 30–60 μg of protein loaded per lane for olfactory neuroblast or brain homogenates and 0.2 μg of purified NSE (Polysciences). Following transfer to nitrocellulose or immobilon membrane (Millipore), blots were incubated for 1 hour in PBS containing 5% nonfat dry milk and then with primary antibodies overnight at 4°C. Reactivity was visualized using phosphatase-coupled secondary antibody (Jackson) using conditions as described for immunocytochemistry. NBT-BCIP was used to visualize the reaction. The dilutions of primary antibodies were: anti-NSE 1 to 2000 (Polysciences), anti-NCAM 1 to 20 (Biodesign, Inc.), GFAP 1 to 100, anti-neurofilament heavy chain 1 to 1000 (Sigma), anti-keratin 1 to 1000 (Dako), anti-vimentin 1 to 500 (gift from D. Jacobowitz), anti-growth-associated protein-43 1 to 1000 (gift from L. I. Benowitz), and anti-OMP 1 to 500.

### Polymerase chain reaction

RNA was isolated from cell cultures as described previously (Chomczynski, 1987). Dishes measuring 35 or 10 mm were incubated 1 minute in 0.5 ml of a lysis buffer consisting of 4 M guanidine thiocyanate, 0.5% sarcosyl, and 50 mM β-mercaptoethanol. Then 37 μl of 3 M sodium acetate, pH 5.4, was added, and a phenol chloroform extraction was performed using 0.5 ml water-saturated phenol and 0.1 ml chloroform. RNA was precipitated from the aqueous phase by addition of 0.5 ml isopropanol and incubating at −20°C for 1 hour. After centrifugation, the pellet was washed two times with 70%

ethanol and dried down. The RNA was taken up in 10 μl $H_2O$ and cDNA was synthesized using 200 units MMLV reverse transcriptase (BRL), buffer, and 10 μM random hexamers as primers in 20 μl final volume. Primers for polymerase chain reaction were directed against the human neurofilament heavy chain: NF2021-AAACTGCCCCCCTCC-TTTC and NF2362-ACATCCAGACCTCCCC-TGC. Five microliters of cDNA mixture, 250 ng each primer, and $1 \times 10^5$ cpm end-labeled NF2021 was used for 24 cycle reactions and 1 μl of cDNA mixture without labeled primer was used for 45 cycle reactions. The program consisted of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, using PCR reaction buffer (Cetus) and 200 μM dNTPs. Following amplification, overlayed mineral oil was removed by extraction twice with chloroform, the samples were dried down, resuspended in 10 μl $H_2O$, sample buffer was added, and the samples were run on an 8% acrylamide or 2% agarose gels. For autoradiography, following electrophoresis, the gel was dried down and exposed.

### cAMP odorant assays

The odorant mixture contained equimolar concentrations of citralva (3,7-dimethyl-2,6-octadiene-1 nitrile), coniferan (2-*tert*-pentylcyclohexenol acetate), β-ionone [4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one], geranyl acetate (3,7-dimethyl-2,6-octadiene-1-yl acetate) (International Flavors and Fragrances), and amyl acetate (Aldrich). Cells were grown in 24-well plates (16-mm well diameter). All points represent quadruplicate determinations. Prior to addition of odorant, cells were preincubated with Coon's modified Ham's F-12 medium, supplemented with 0.1 mM $CaCl_2$, 20 mM HEPES (pH 7.4), and 0.5 mM 3-isobutyl-1-methylxanthine for 10 minutes at 37°C. After removal of this, an equal volume of odorant mixture in the same medium in the concentrations indicated was added for 5 minutes longer. The reaction was stopped by addition of 3% perchloric acid. cAMP was measured using a cAMP assay kit (Diagnostic Products Inc). Nonstimulated cAMP levels were 3.0 ± 0.5 pmol/well.

### Calcium photometry

Cytosolic free calcium concentration was monitored in individual cells with the fluorescent $Ca^{2+}$ indicator, Indo-1 (Mollard et al., 1989). Cells, grown on a glass cover slip coated with RBM, were incubated with 10 μM Indo-1 AM and 0.06% pluronic F127 (Molecular Probes) for 30 minutes at room temperature. Following washing, the cells were incubated in PBS containing 2 mM $CaCl_2$. Indo-1 fluorescence was recorded, while odorant mixture (1 μM), which was identical to that used in the cAMP assays, was pulsed onto the cells through a glass capillary pipet. The results shown are for one cell; however, substantially similar results were obtained for other cells as well. The dual-emission microspectrofluorimetric apparatus consisted of a Nikon Diaphot inverted microscope fitted for epifluorescence. Excitation light was filtered at 360 nm. Calcium concentration was estimated from the ratio of emission light recorded at 410 nm and 490 nm with two separate photometers.

## Results

### Cell culture

Olfactory epithelium was obtained from 10 cadavers at autopsy. The age and postmortem intervals for the donors is described in Table 1. The cell lines used for the biochemical and physiologic studies reported in this communication were those of 90-1 and 90-8. Substantially similar results, however, have been obtained with all the cell lines. The cell types that grew out were morphologically heterogeneous and included cells resembling those of olfactory neurons, and epithelial, Schwann, and mucosal cells. However, only one cell type, the neuroblasts, maintained the ability to continue to proliferate beyond 2–3 weeks (Fig. 1A). Other tissues taken from the same adult human donors at autopsy, such as samples from olfactory bulb, substantia nigra, abdominal epithelium or aorta, yielded no continuously proliferating cells when grown under the same conditions. Part of the selectivity of this method may be due to age, because tissue taken from em-

**Table 1.** Sources of Olfactory Epithelium[1]

| Cell line label | Age | Postmortem interval (hours) |
|---|---|---|
| 1390 | 29 | 14 |
| 1394 | 30 | 11 |
| 1401 | 36 | 11 |
| 1402 | 35 | 10 |
| 90-1 | 57 | 8 |
| 90-4 | 34 | 9 |
| 90-5 | 62 | 9 |
| 90-8 | 45 | 7 |
| 90-9 | 23 | 10 |
| 90-10 | 30 | 8 |
| 90-11 | 19 | 10 |
| Mean values: | 36.4 ± 13.3 | 9.7 ± 1.9 |

[1] All of the olfactory epithelial explants were obtained at autopsy. The postmortem interval is defined as the time from reported death until the tissue sample was put into L-15 medium. The cause of death for all of the donors was trauma.

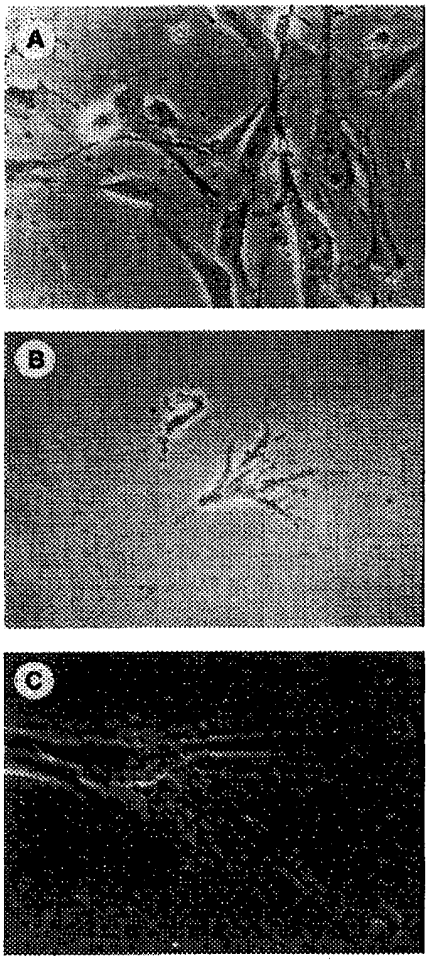


**Fig. 1.** Morphology of cultured olfactory neuroblasts. The main population of proliferating cells are asymmetric cells that extend processes (**A**). When grown imbedded in RBM, the cells often branch extensively, send out filaments resembling cilia, and occasionally develop multiple knobby spines (**B**). (**C**) Cilia-like filaments at higher magnification (40×).

bryonic, but not adult, rat olfactory bulb and hippocampus did yield proliferating cells, many of which were positive for glial antigens by immunocytochemistry.

The presumptive neuroblasts were isolated based on morphology and subsequently expanded in culture. A number of neuroblast colonies were subjected to monoclonal selection, with the colonies selected based on their morphology, and the populations expanded. For immunocytochemical and morphological analyses, both clonal and nonclonal colonies were examined. For biochemical studies, pure neuroblast populations were used, but the colonies were not subjected to the step of monoclonal selection. The cloning step was omitted in order to allow the generation of larger numbers of cells that would survive for more passages in culture. The clonal colonies and nonclonal colonies looked and behaved identically by all morphologic and immunochemical criteria tested. With the exception of an increase in size as the cells senesce, the morphologies of all cell lines, both clonal and nonclonal, have been constant (10–30 passages depending on the colony). Growth rates of the cell strains varied with serum lot and donor, but doubling times tended to be at least 48 hours. Cells from the older donors grew more slowly, while cells from younger adults maintain a robust growth rate for 3–6 months (20 or more passages). Karyotypes revealed a diploid complement with no evidence of major translocations.

**Cell morphology**

Under normal growth conditions, the majority of cells are asymmetric cells that extend processes whose length increases with slower growth rates (Fig. 1A). A second group of cells more nearly resembles the differentiated olfactory neurons seen in vivo. These cells exhibit swollen knobs at the end of short processes, often with projections resembling cilia-like dendritic vesicles (Fig. 3C and D below). Bromodeoxyuridine incorporation experiments resulted in much less staining of these cells than of the putative stem cells, suggesting that these cells divide more slowly than the putative stem cells. Because these cells continue to be present over multiple generations, it appears likely that they are generated from the stem cells and may represent a more highly differentiated state.

The morphological differentiation of the cells can be influenced by a number of factors. When they are grown embedded within reconstituted basement membrane matrix (RBM) such as during the initial plating when they first grow from the tissue or when they are plated directly in RBM, the cells often exhibit extensive branching on one side of the cell and extension of filaments resembling cilia on the other side (Fig. 1B and C). There are occasionally multiple knobby spines that can be seen along these branched processes (Fig. 1B). In normal "flat" tissue culture conditions, slower growth rates resulted in more extensive process production for many of the cells. Incubation in protein-free medium (culture in medium without serum or extracts) produced a decrease in the caliber of the processes and an increase in process outgrowth over a 2-week time course (Fig. 2A). The extent of process outgrowth could be dramatically increased by addition of pharmacologic agents such as the combination of

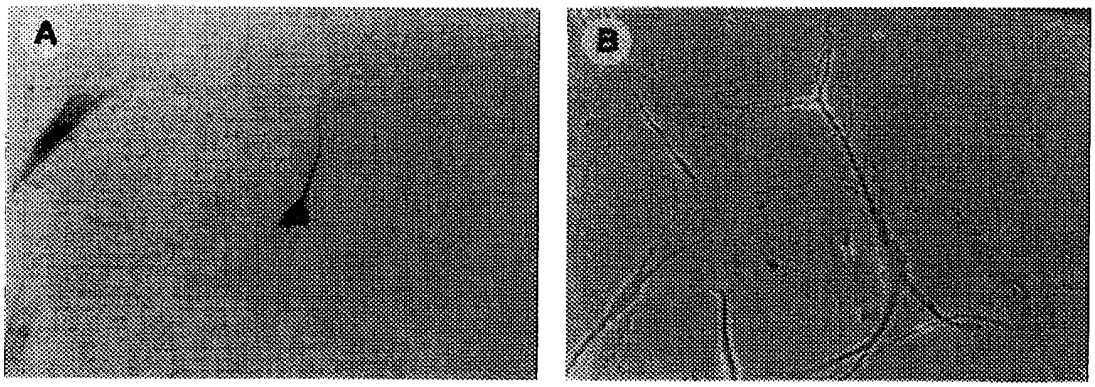


**Fig. 2.** (A) Cells grown for four days in protein-free medium and stained with anti-NSE. (B) Process outgrowth from cells incubated 48 hours in 4506 medium supplemented with 10 ng/ml nerve growth factor, 0.5 mM db-cAMP, and 0.5 mM 3-isobutyl-1-methylxanthine.

0.5 mM dibutyril-cyclic AMP (db-cAMP) and the phosphodiesterase inhibitor isomethylbutylxanthine (IBMX, 0.5 mM) with or without the added hypothalamus and pituitary extracts (Fig. 2B). Under this paradigm process outgrowth could be observed between 24 and 48 hours after addition of the agents. Supplementation of the cocktail with nerve growth factor produced more extensive process outgrowth.

**Immunohistochemistry**

All the clonal cell strains studied stained with antibodies to the neuronal antigens: neuron-specific enolase, neurofilament protein (heavy and light chain, only staining for heavy chain shown), calcitonin-gene-related peptide (CGRP), and olfactory marker protein (staining for neurofilament and OMP are shown in Fig. 3A and D, respectively). No staining was seen for NCAM. Staining for the glial protein, glial fibrillary acidic protein (GFAP), was also evident (Fig. 3B). The staining with these markers was uniform in nature, and double-staining studies did not reveal cells that stained more intensely for the neuronal markers than for the GFAP.

**Immunochemistry**

Immunohistochemical staining was verified by immunoblot analysis. Immunoreactive bands of correct mobilities were detected for NSE, neurofilament (heavy and light chain, only staining for the heavy chain is shown), OMP, vimentin, keratin, growth-associated protein (GAP43), and GFAP (Fig. 4). The neurofilament bands comigrated with corresponding bands present in human brain and were absent in lymphoblast homogenates (Fig. 4A). The OMP band is shown comigrating with an OMP present in homogenate from rat olfactory epithelium, which has the same migration coefficient as human OMP (Fig. 4B). Immunoreactive bands of this mobility were absent in lymphblast homogenate and human brain homogenate. The NSE band shown comigrated with that of purified human NSE (Fig. 4C). Immunoblots with anti-NCAM revealed a single 120-kDa band (data not shown). Vimentin was found to be present both in the neuroblasts and in rat olfactory epithelium (Fig. 4D). Keratin and GAP43 were also found to be present (Fig. 4E and F). GFAP was also detected and showed a pattern similar to that of adult human brain (Fig. 4G).

Because in vivo only occasional olfactory neurons express neurofilament protein, we sought further proof for the identity of neurofilament by detecting neurofilament transcript. ON total RNA was converted to cDNA and amplified by polymerase chain reaction (PCR) using primers for the neurofilament heavy chain. As shown in Fig. 5, neurofilament product of the expected size of 363 bp was seen after 45 rounds of amplification. The 363 neurofilament product could also be detected after 24 rounds of amplification using $^{32}$P-labeled primer with $10^5$ cpm/reaction. Lymphoblast cDNA yielded no neurofilament product.

**Cellular physiology**

We have examined the functional activity of these cell strains by measuring their response to odorants. Odorant-dependent cyclic AMP accumulation was assayed after incubating live cells with a mixture of odorants known to elicit cAMP responses (Coon et al., 1989; Sklar et al., 1986). The cell strain 90-1, which showed a particularly strong cAMP response, had a maximal accumulation of the levels of cAMP at 0.1 μM odorant (Fig. 6A). cAMP levels

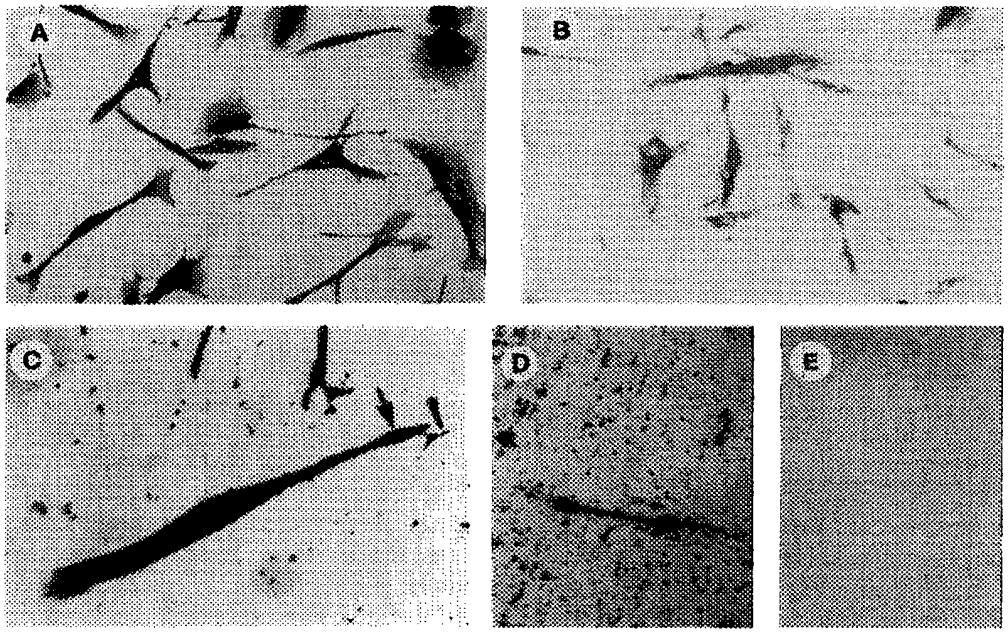


**Fig. 3.** Immunohistochemical staining was with antibodies to neurofilament (A), GFAP (B), CGRP (C), and OMP (D). Omission of primary antibody resulted in no detectable staining (E). Occasional neuroblasts exhibited an olfactory vesicle-like terminal swelling at the end of a short process (D, arrow).

could also be increased approximately sixfold by incubation of the cells with 10 μM forskolin or isoproteronol. Nonneuronal cells did not respond to odorants. Odorant-dependent increases in intracellular calcium were detected using Indo-1 fluorescence (Fig. 6B). Although the cells were not spontaneously active in 4506 medium, application of 1 μM odorant mixture produced an increase in intracellular calcium in 5–10% of the cells. A calcium spike with a rapid rise time and a duration of 10 seconds was elicited, as shown for a cell from cell stain 90-8 (Fig. 6B). Repeated application of odorant produced lessened responses similar to the phenomenon of habituation seen in vivo.

## Discussion

We have been able to culture ON from adult human autopsy material. The conclusion that these cultured cells are neuronal derives from three lines of evidence: morphological, biochemical, and physiological. Morphologically, the cells resemble cultures of rat olfactory neurons and neuroblasts, as has been previously described (Coon et al., 1989; Pixley and Pun, 1990; Schubert et al., 1985). The cells extend processes and have filaments resembling cilia, as do olfactory neurons. Biochemically, the cells exhibit characteristics of olfactory neurons in that they express neuronal proteins including neuron-specific enolase, olfactory marker protein, neurofilament protein, and NCAM. The presence of neurofilament protein has been further confirmed by the demonstration of neurofilament transcript. Physiological evidence supports the identification of these cells as olfactory neurons with the demonstration of odorant-dependent increases in cAMP and intracellular calcium. The cAMP response occurred at submicromolar levels, and the calcium response occurred with 1 μM pulses of odorant. Both of these concentrations are well below the levels at which odorants elicit nonspecific responses in nonneuronal cells. Together, this combination of morphological, biochemical, and physiological data indicate that cells with the properties of olfactory neuroblasts can be propagated in cell culture from explants of human olfactory epithelium taken at autopsy.

The presence of OMP in these cells provides strong evidence of their olfactory nature and suggests the presence of mature olfactory neurons (Keller and Margolis, 1975; Calof and Chikaraishi, 1989). The expression of NCAM provides further support for cells differentiated beyond the precursor stage. The detection of OMP described in this paper contrasts with previous work done by other investigators (Coon et al., 1989; Pixley and Pun, 1991). The reason for the presence of OMP in this

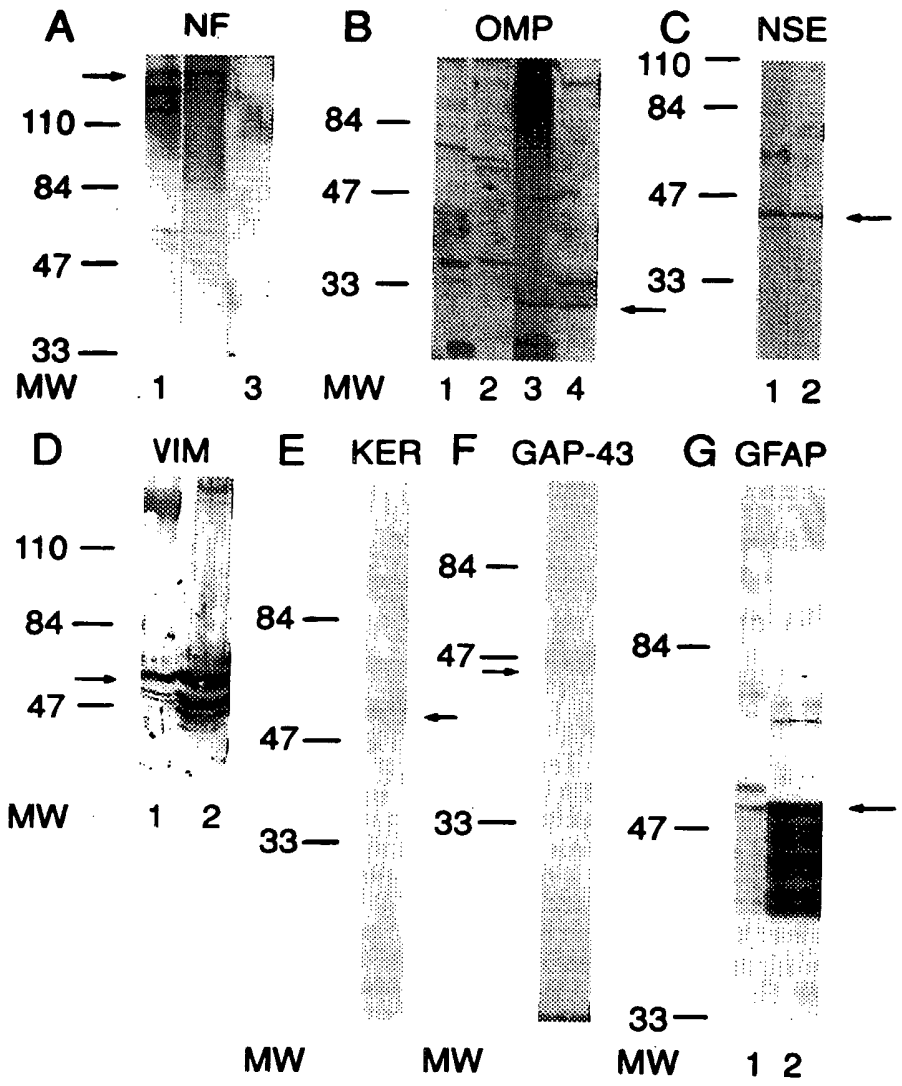


**Fig. 4.** Immunoblot analysis of crude homogenates of olfactory neuroblasts. **(A)** Neurofilament antibodies against the heavy neurofilament chain (NF) recognized gave similar banding pattern for homogenates of human brain (lane 1) and ON (lane 2). No reactivity was seen in lymphoblast homogenates (lane 3). **(B)** Antibodies to OMP yielded a band of approximately 20 kDa in ON homogenate (lane 3) that comigrated with a similar band present on homogenate from rat olfactory epithelium (lane 4). Homogenates of human brain (lane 2) and lymphoblasts (lane 1) yielded no immunoreactive bands in this region. **(C)** An anti-NSE reactive band present in ON homogenate (lane 2) comigrated with purified human NSE (lane 1). **(D)** Vimentin (VIM) was clearly visible in ON homogenate (lane 1), and it comigrated with vimentin present in human brain (lane 2). **(E and F).** Immunoreactive bands of the appropriate mobility were seen for keratin (E, KER) and GAP43 (F), the latter stained using a 100 k x g pellet from ON homogenate. **(G)** The pattern for GFAP staining in ON homogenate (lane 1) showed a banding pattern similar to that in human brain (lane 2). Elimination of primary antibody from the the reaction resulted in no reactivity with the bands in question.

system may be due to the tissue source. Previous investigations have focused on cells taken from neonatal animals (Coon et al., 1989; Pixley and Pun, 1991). It is possible that generation of the cells from adult tissue selects for a somewhat more mature population of cells in that the dividing time is slower than it is in cells taken from neonatal rats. Often, as these cultures senesce, the dividing time slows

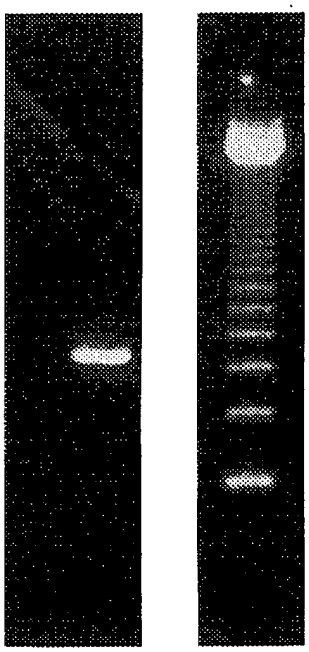

Fig. 5. PCR amplification of a 363 nucleotide neurofilament product using cDNA from ON cultures. The molecular weight standard is a 123 bp ladder.

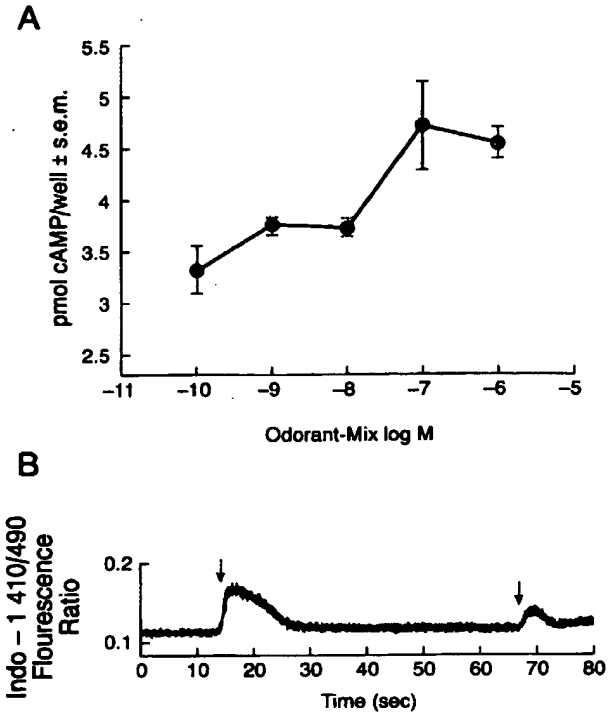


Fig. 6. Odorant-induced increase in cAMP (A) or intracellular calcium (B). The arrows in B indicate the time of application of a 100-msec pulse of odorant.

down to the point where individual cells may be nonmitotic for weeks. Under these conditions, differentiation may proceed to a point where OMP is expressed.

Neurofilament is normally only sporadically expressed in the olfactory epithelium in vivo but is abundantly expressed in these cells in vitro (Schwob et al., 1986). The discrepancy in the extent of neurofilament expression between the in vivo and in vitro states is an interesting finding that may relate to the function of this protein in olfactory neurons. The fact that ON can express neurofilaments in vivo at all demonstrates that these cells do have the capacity to express neurofilament. Therefore, the abundant expression of neurofilament in vitro, although surprising, is not out of character. The neurofilament expression in vivo may be related to the extension of new axons as the olfactory neurons regenerate. In this context, the sporadic expression of neurofilament in vivo is consistent with the documented regeneration rate of ON, which is 1–3% (Grazeidei, 1973). In vitro, however, the cells are constantly proliferating and extending processes. In this state the cellular dynamics are more similar to a state of rapid and prolific regeneration in vivo, hence neurofilament may be more abundantly expressed. GAP43 is a calcium-binding protein normally found in growth cones of neurons (Benowitz and Routtenberg, 1987). The presence of GAP43 in these cultures provides further evidence for the existence of growing neuronal processes.

In addition to neuron-specific proteins, we have also detected several proteins that, although not neuron specific, are characteristic of ON cells. These proteins include vimentin, CGRP, and keratin. Vimentin is found in a wide variety of cells in vivo, including neurons in the olfactory epithelium. Typically, vimentin is found in immature cells (Schwob et al., 1986). Vimentin and neurofilament do not commonly coexist in cells; however, colocalization has been detected in neurons of the developing human retina as well as in the rat olfactory epithelium (Stagaard and Mollgard, 1989; Schwob et al., 1986). The coexistence of vimentin and neurofilament in these cells is therefore within the expected biological parameters of the system. CGRP has been detected previously both in cultured olfactory neurons and in the olfactory epithelium in vivo (Denis-Donini, 1980; Finger et al., 1990). Finally, the presence of keratin is consistent with the epithelial origin of the cells and is also consistent with similar findings in vivo (Calof and Chikaraishi, 1989). Because keratin is normally found in basal

cells of the olfactory epithelium, its presence suggests that immature cells do exist in the cultures.

Although the data strongly support the characterization of these cells as neuronal, there are results that have yet to be explained. A principal piece of data that is confusing is the presence of GFAP. Detection of GFAP in cultured olfactory neurons has been described previously (Schubert et al., 1985) but is nonetheless puzzling. The coexistence of GFAP and neuronal markers has been documented in other developing or incompletely differentiated systems. For instance, medulloblastoma cells can express GFAP or neurofilament protein (Zeltzer et al., 1990). Neuronal stem cells may also be able to express both glial and neuronal proteins (Omlin and Waldmeyer, 1989). Hence, the coexpression of neuronal and glial proteins in the cultured ON suggests that these cells are incompletely differentiated and may be bipotential. Because mature neurons are generally felt to be incapable of cell division, this incomplete differentiation may be beneficial in that it may facilitate the proliferation of these cells.

The immunochemical data suggest that the cells in these cultures express a heterogeneity of differentiation states. The presence of at least two populations of cells, even in monoclonal colonies, provides further support for this suggestion. If the bulbous cells, which constitute only 5–10% of the cells, are more differentiated cells, then the majority of cells would be incompletely differentiated. The low abundance of bulbous cells (5–10%) is consistent with the abundance of responders in the calcium fluorometry study. The nonresponding cells might be less differentiated. Alternatively, the nonresponders may be capable of an odorant response but respond to different odorants. Recent work has identified molecules that appear to be involved in the odorant response, and in rat there appear to be at least 20 different odorant receptors (Bakalyar and Reed, 1990; Buck and Axel, 1991; Dhallan et al., 1990; Lazard et al., 1991). Currently, probes for these molecules are only available for the rat odorant system, but as the technology evolves, such probes should become available for the human system as well. It will be of interest to examine the expression of these and other odorant-related molecules on a cellular level. Such studies may enable the study of the generation of diversity of the odorant receptors, much as the diversity of the antibody response has been studied in the immune system.

Understanding the regulation of the differentiation state in the cells is of great potential. Future research in this direction is particularly relevant in the context of these human cell lines because maximization of the neuronal character of the cells may facilitate the creation of cultures that express traits relevant to neurosphychiatric illness. In this context, modulating the differentiation state is an important avenue of further research. Agents that mimic cAMP clearly alter the morphology of the cells. Other agents, such as growth factors or neuropeptides, may also regulate the morphology and, perhaps, differentiation state. This system may be analogous to hematopoeitic stem cell cultures prior to the discovery of colony-stimulating factors and interleukins, which effect more complete differentiation. The early hematopoeitic stem cell cultures were heterogeneous and did not differentiate completely (Dexter, 1989). In the same manner that colony-stimulating factor promotes the differentiation of hematopoeitic stem cells, there may be factors that will promote the differentiation of the olfactory neuroblasts.

The ability to obtain neuronal cells from humans has important clinical implications and may be the most valuable aspect of this work. These cells can been obtained after a postmortem interval of up to 13 hours. In addition to acquiring tissue at autopsy, it may be possible to acquire tissue at biopsy. The olfactory epithelium is readily accessible to biopsy; hence biopsy may become an important method of tissue acquisition (Lovell et al., 1982). These cultures of ON offer an alternative to other cell systems that have been used to study neurospychiatric illness, such as the HCN cell line, neuroblastomas, lymphoblasts and fibroblasts (Ronnett et al., 1990). The HCN and neuroblastoma cell lines are derived from different regions than the ON; hence these cell lines may express some neuronal traits not present in the ON cultures. On the other hand, because many neuropsychiatric illnesses are not expressed until adulthood, the capability of growing ON cultures from adults allows the generation of neuronal cell strains from individuals who already have clinically diagnosed illnesses. This is in contrast to the HCN and neuroblastoma cell lines, which are uncommon and generally come from children. The ability to generate cell lines from targeted individuals is similar to the advantages present in studying lymphoblasts, fibroblasts, or platelets. However, unlike these mesenchymal cells, cultures of olfactory neuroblasts are neuronal. Being neuronal, they may express physiologic processes important to the pathophysiology of neuropsychiatric illnesses that are absent in nonneuronal cells. In vivo, olfactory neurons develop some of the pathology of Alzheimer's disease in that they express immunoreactivity with the antibody Alz-50, an early marker of neurofibrillary pathology (Talamo et al., 1989; Wolozin

et al., 1986). We are currently acquiring samples from donors afflicted with Alzheimer's disease in order determine whether cultured olfactory neuroblasts express elements of the pathophysiology of Alzheimer's disease.

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## References

Bakalyar, H.A., Reed, R.R. (1990). Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science 250:1403–1406

Benowitz, L.I., Routtenberg, A. (1987). Trends Neurosci. 10:527–531

Buck, L., Axel, R. (1991). A novel multigen family may encode odorant receptors: A molecular basis for odorant recognition. Cell 65:175–187

Calof, A.L., Chikaraishi, D.M. (1989). Analysis of neurogenesis in a mammalian neuroepithelium: Proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3:115–127

Chamczynski, P., Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162:156–159

Coon, H.G., Curcio, F., Sakaguchi, K., Brandi, M.L., Swerdlow, R.D. (1989). Cell Cultures of neuroblasts from rat olfactory epithelium that show odorant responses. Proc. Natl. Acad. Sci. U.S.A. 86:1703–1707

Denis-Donini, S. (1989). Expression of dopaminergic phenotypes in the mouse olfactory bulb induced by the calcitonin gene-related peptide. Nature 339:701–703

Dexter, T.M. (1989). Haematopoietic growth factors. Br. Med. Bull. 45:337–349

Dhallan, R.S., Yau, K., Schrader, K.A., Reed, R.R. (1990). Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature 347:184–187

Finger, T.E., St. Jeor, V.L., Kinnamon, J.C., Silver, W.L. (1990). Ultrastructure of substance P and CGRP immunoreactive nerve fibers in the nasal epithelium of rodents. J. Comp. Neurol. 294:293–305

Goldstein, N.I., Quinn, M.R., Teeter, J.H. (1986). Differentiation of a cell line from olfactory epithelium is induced by dibutyryladenosine 3′,5′-cyclic adenosine monophosphate and 12-O-tetradecanoyl phorbol-13-acetate. Brain Res. 370:205–214

Graziadei, P.P.C. (1973). Cell dynamics in the olfactory mucosa. Tissue Cell 5:113–131

Keller, A., Margolis, F.L. (1975). Immunological studies of the rat olfactory marker protein. J. Neurochem. 24:1101–1106

Kischer, C.W., Leibovitz, A., Pindur, J. (1989). The use of a transport medium (L15M15) for bulk tissue storage and retention of viability. Cytotechnology 2:181–185

Lazard, D., Zupko, K., Poria, Y., Nef, P., Lazarovits, J., Horn, S., Khen, M., Lancet, D. (1991). Odorant signal termination by olfactory UDP glucoronosyl transferase. Nature 349:790–793

Lovell, M.A., et al. (1982). Biopsy of human olfactory mucose. Arch. Otolaryngol. 108:247–249

Mollard, P., Guerineau, N., Audin, J., Dufy, B. (1989). Measurement of $Ca^{2+}$ transients using sumultaneous dual-emission microspectrofluorimetry and electrophysiology in individual pituitary cells. BBRC 164:1045–1052

Omlin, F.X., Waldmeyer, J. (1989). Differentiation of neuron-like cells in cultured rat optic nerves: A neuron or common neuron-glia progenitor? Dev. Biol. 133:247–253

Pixley, S.K., Pun, Y.K. (1990). Cultured rat olfactory neurons are excitable and respond to odors. Dev. Brain Res. 53:125–130

Ronnett, G.V., Hester, L.D., Nye, J.S., Connors, K., Snyder, S.H. (1990). Human cortical neuronal cell line: Establishment from a patient with unilateral megalencephaly. Science 248:603–605

Schubert, D., Stallcup, W., LaCorbiere, M., Kidokoro, Y., Orgel, L. (1985). Ontogeny of electrically excitable cells in cultured olfactory epithelium. Proc. Natl. Acad. Sci. U.S.A. 82:7782–7786

Schwob, J.E., Farber, N.B., Gottlieb, D.I. (1986). Neurons of the olfactory epithelium in adult rats contain vimentin. J. Neurosci. 6:208–217

Sklar, P.B., Anholt, R.R.H., Snyder, S.H. (1986). The odorant-sensitive adenylate cyclase of olfactory receptor cells. J. Biol. Chem. 261:15538–15543

Stagaard, M., Mollgard, K. (1989). The developing neuroepithelium in human embryonic and fetal brain studied with vimentin-immunocytochemistry. Anat. Embryol. 180:17–28

Talamo, B.R., et al. (1989). Pathological changes in olfactory neurons in patients with Alzheimer's disease. Nature 337:736–739

Wolozin, B.L., Pruchnicki, A., Dickson, D.W., Davies, P. (1986). A neuronal antigen in the brains of Alzheimer patients. Science 232:648–650

Zeltzer, P.M., Bodey, B., Marlin, A., Kemshead, J. (1990). Immunophenotype profile of childhood medulloblastomas and supratentorial primitive neuroectodermal tumors using 16 monoclonal antibodies. Cancer 66:273–283